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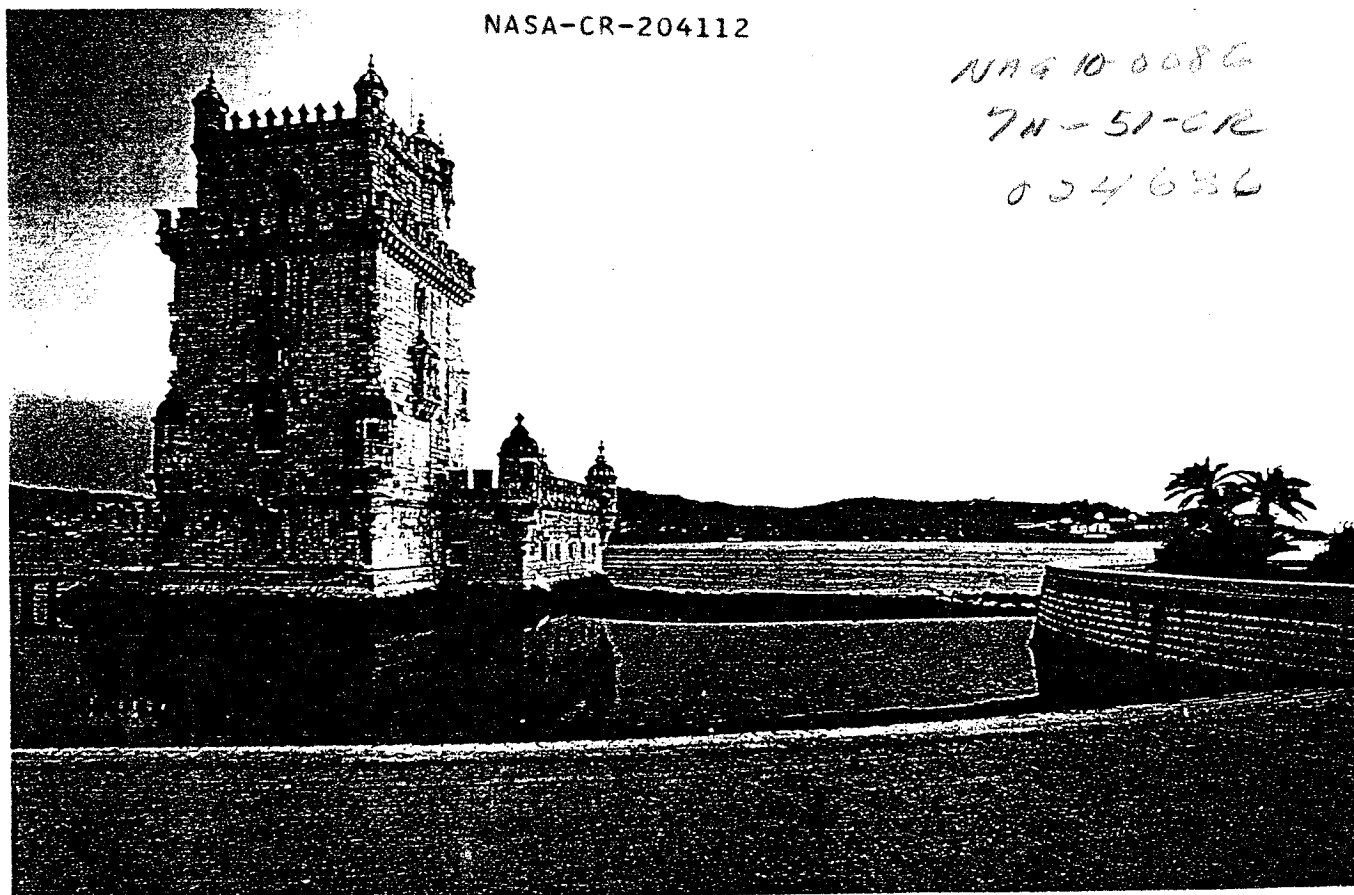Fundação Calouste Gulbenkian
LISBOA, PORTUGAL

13-16 Juillet 1992
July 13-16, 1992

COMPTE-RENDU
PROCEEDINGS

TOME I - Volume 16

NASA-CR-204112

Elucidation of the Enzymatic Conversions in Lignan Biosynthesis: (+)-Pinoresinol Synthase, (+)-Pinoresinol and (+)-Lariciresinol Reductase
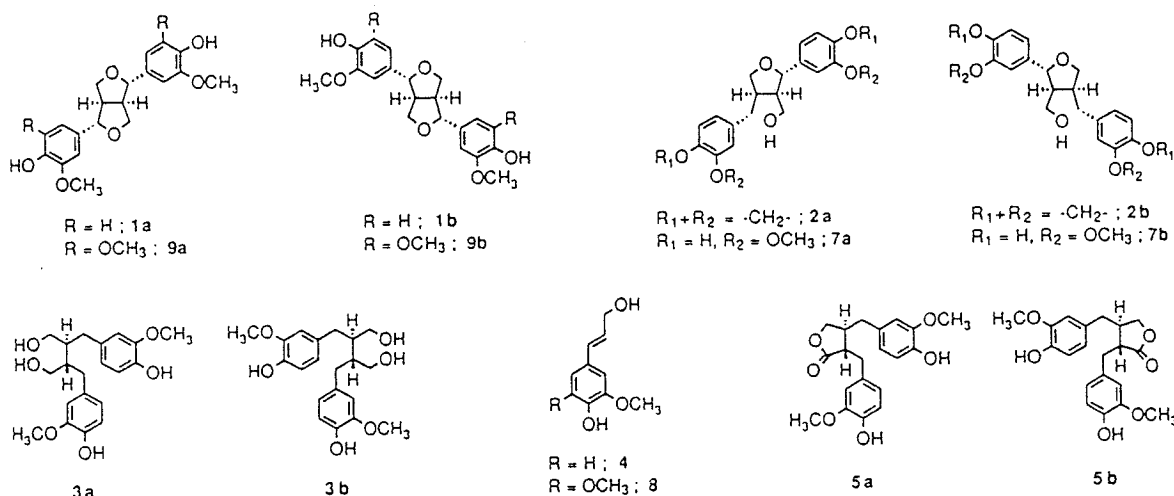
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Summary

(+)-Pinoresinol synthase, an enzyme catalyzing the highly unusual stereoselective coupling of two achiral *E*-coniferyl alcohol molecules, has been discovered for the first time; this discovery is the first example of *stereoselective* coupling in phenylpropanoid metabolism. The enzyme is present in the "insoluble residue" from *Forsythia* species, obtained following removal of readily soluble enzymes. Interestingly, this preparation is capable of engendering(+)-pinoresinol formation, even in the absence of exogenously supplied cofactors; however, enzymatic activity is stimulated when malate and NAD are added. By contrast, the soluble enzyme preparation from *Forsythia intermedia* contains a NAD(P)H-dependent reductase (or reductases) which converts (+)-pinoresinol into (+)-lariciresinol, and subsequently transforms the latter into (-)-secoisolariciresinol; significantly, the corresponding (-)-antipodes of pinoresinol or lariciresinol do not serve as substrates for this reduction. These enzymes are highly unusual since they apparently catalyze direct benzylic ether reduction (or a quinone methide intermediate derived thereof) at their active sites.

Introduction

The biologically active lignans and neolignans are a class of phenylpropanoids with various functions *in vivo* which include plant defense (DAVIN and LEWIS, 1992), e.g., dehydrodiisoeugenol is partly responsible for the bactericidal properties of *Myristica fragrans* Houtt (HATTORI *et al.*, 1986), magnolol is a phytoalexin present in stressed *Cercidiphyllum japonicum* plants (TAKASUGI and KATUI, 1986), sesaminol is an antioxidant in sesame seeds (FUKUDA *et al.*, 1986), and secoisolariciresinol is considered to be an active principle conferring decay resistance in parana pine (*Araucaria angustifolia*) (ANDEREGG and ROWE, 1974). Lignans and neolignans also display numerous pharmacological properties in mammalian species (ADLERCREUTZ, 1984). For example, the observed reduction in incidence rates of breast and prostate cancers with individuals on high fiber diets is apparently positively correlated with formation of the "mammalian" lignans, enterolactone and enterodiol; these are formed in the intestine by metabolism of dietary plant substances.

R = H : 1a
R = OCH₃ : 9a

R = H : 1b
R = OCH₃ : 9b

R₁+R₂ = -CH₂- : 2a
R₁ = H, R₂ = OCH₃ : 7a

R₁+R₂ = -CH₂- : 2b
R₁ = H, R₂ = OCH₃ : 7b

3a

3b

R = H : 4
R = OCH₃ : 8

5a

5b

Lignans typically occur enantiomerically pure in nature, although the specific optical isomer can vary with the plant species. For example, *Forsythia suspensa* and *Juniperus sabina* accumulate (+)-pinoresinol 1a (UMEZAWA *et al.*, 1990b) and (+)-dehydrosesamin 2a (SAN FELICIANO *et al.*, 1990), respectively, but not the corresponding (-)-antipodes. By contrast, (-)-pinoresinol 1b and (-)-dihydrosesamin 2b are found in *Daphne tangutica* (ZHUANG *et al.*, 1982). A focus in this laboratory has been to establish the nature of the enzymatic transformations involved in lignan formation, with a particular emphasis placed upon the stereoselectivity and enantiospecificity of such conversions. In this context, we established that cell-free preparations from *Forsythia intermedia*

(+)-enantiomeric 1a form was observed to rapidly decline with time until essentially only the (-)-antipode 1b (> 90%) remained. [It must be cautioned that this evidence is only based on the radiochemical elution data corresponding to known retention times of (+)- and (-)-pinoresinols 1a/1b.] Lastly, as can be seen from the lower trace (Fig. 2), the apparent synthesis and turnover of (+)-pinoresinol 1a and (-)-secoisolariciresinol 3b is also accompanied by the formation of another enzymatic (radiolabeled) product (discussed below).

Figure 2. Time course of (-)-secoisolariciresinol 3b and (±)-pinoresinol 1a/1b synthesis and accumulation. *F. intermedia* cell-free extracts were incubated with [8-^{14}C]coniferyl alcohol 4 in the presence of H_2O_2 (0.4 mM) and NAD(P)H (4 mM). X = (±)-pinoresinols 1a/1b; ■ = (+)-pinoresinol 1a; ☐ = (-)-pinoresinol 1b; O = (-)-secoisolariciresinol 3b and ▲ = (+)-lariciresinol 7a.
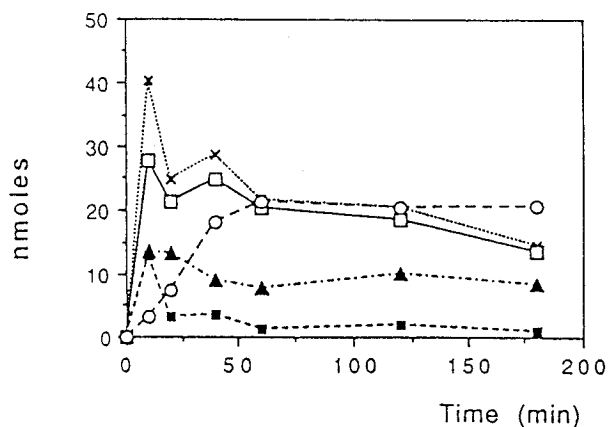
In order to establish beyond any reasonable doubt that the enzymatic product accumulating in the extract was (-)-pinoresinol 1b, the *F. intermedia* cell-free preparation was incubated with [9-^{2}H$_2$,OC^{2}H$_3$]coniferyl alcohol 4, H_2O_2 (0.4 mM) and NAD(P)H (4 mM) for 2 h, following which the resulting enzymatic products were subjected to purification by reversed phase and chiral column HPLC. Once again, the major product formed (> 90%) had a retention volume corresponding to the (-)-antipode 1b. Mass spectroscopic analysis of the enzymatic product and comparison with authentic (±)-pinoresinols 1a and 1b established it to be (-)-[9,9'-^{2}H$_2$,OC^{2}H$_3$]pinoresinol 1b (data not shown).

Taken together, these observations strongly suggested that the *F. intermedia* cell-free extracts initially catalyzed the H_2O_2-dependent, non-specific, peroxidase-catalyzed, coupling of two molecules of *E*-coniferyl alcohol 4 to yield both (+)- and (-)-pinoresinols 1a and 1b, with [8R,8'R] and [8S,8'S]quinone methides 6a and 6b as possible intermediates. But the (+)-pinoresinol 1a so formed was then apparently converted into (-)-secoisolariciresinol 3b via an enantiospecific NAD(P)H-dependent enzymatic reduction. Thus, to establish whether this was occurring, both (+)- and (-)-pinoresinols 1a and 1b were incubated with the *F. intermedia* cell-free preparation in the presence of 0.4 mM H_2O_2, 0.4 mM H_2O_2/4 mM NAD(P)H and 4 mM NAD(P)H as cofactors, respectively. With H_2O_2 alone as cofactor, it was found that no (-)-secoisolariciresinol 3b formation occurred. Conversely, with 4 mM NAD(P)H as cofactor, depletion of the (+)-pinoresinol enantiomer 1a occurred rapidly with concomitant formation of both (-)-secoisolariciresinol 3b and the unknown enzymatic product previously observed in the radiochemical study; as before, the (-)-pinoresinol 1b content remained essentially unchanged during the assay. [Similar findings were also observed when both NAD(P)H and H_2O_2 were used as cofactors.]

Attention was next focused upon identification of the unknown pinoresinol-derived enzymatic product observed during (-)-secoisolariciresinol 3b formation. In this regard, the most likely candidate as an intermediate in the conversion of (+)-pinoresinol 1a to (-)-secoisolariciresinol 3b was (+)-lariciresinol 7a, in a process whereby the furanofuran ring system of (+)-pinoresinol 1a is sequentially reduced. Thus, to establish the identity of the unknown product, (±)-1a/1b, (+)-1a, and (-)-1b-pinoresinols were individually incubated with *F. intermedia* cell-free extracts in the presence of 4 mM NAD(P)H for 120 min. After examination of each assay mixture by reversed phase HPLC, it was established that the unknown product was formed only when (+)-pinoresinol 1a was present. Importantly, the retention volume of the unknown product by reversed phase HPLC was identical to that of authentic lariciresinol 7. Additional proof that the unknown product was lariciresinol 7 was established by UV, mass spectroscopic, and ^{1}H NMR analysis. Subsequent analysis of the enzymatically produced lariciresinol 7 by chiral column HPLC analysis revealed that essentially only the (+)-antipode 7a was formed, i.e., that the reduction of (+)-pinoresinol 1a was highly enantiospecific. In an analogous manner, (±)-7a/7b, (+)-7a, and (-)-7b-lariciresinols were next individually incubated with the *F. intermedia* cell-free preparation in the presence of 4 mM NAD(P)H, where once again essentially only (-)-secoisolariciresinol 3b was formed, provided that (+)-lariciresinol 7a was present in the assay

mixture, i.e., (-)-lariciresinol **7b** did not serve as an effective substrate for conversion into either (+)- or (-)-secoisolariciresinols **3a** or **3b**. Thus, it was concluded that the *F. intermedia* cell-free preparations catalyzed a non-stereoselective H_2O_2-dependent peroxidase-catalyzed coupling of *E*-coniferyl alcohol **4** to afford (±)-pinoresinols **1a** and **1b**, and that the (+)-pinoresinol **1a** so formed underwent enantiospecific reduction to afford (+)-lariciresinol **7a**, which was then further reduced to give (-)-secoisolariciresinol **3b**.

The next question to be addressed concerned pinoresinol **1** formation, since in both *F. intermedia* and *F. suspensa* only the (+)-enantiomer **1a** accumulates (UMEZAWA *et al.*, 1990b). In this regard, it could not be discounted that masking of the desired stereoselective enzymatic activity in the cell-free preparation was occurring as a consequence of competition for substrate (*E*-coniferyl alcohol **4**) by non-specific peroxidase activities resulting in the formation of both antipodes **1a** and **1b**. Accordingly, a significant effort was first carried out to fractionate the enzymes in the *F. suspensa* cell-free preparation in an attempt to detect either a specific enzyme capable of catalyzing the stereoselective coupling of two *E*-coniferyl alcohol **4** molecules to give (+)-pinoresinol **1a**, or (less likely) an enzyme selectively depleting the (-)-antipode **1b**. However, all such efforts failed and attention was next turned to the "insoluble" residue remaining after removal of the soluble enzymes from *F. suspensa* (DAVIN *et al.*, 1992).

Before describing the results obtained with the *F. suspensa* "insoluble" residue, it is important to discuss findings on related topics obtained almost 15 years ago. In these previous seminal investigations, it was reported that "cell walls" from horseradish (*Armoracia lapathifolia* Gilib) ELSTNER and HEUPEL, 1976) were capable of catalyzing the formation of H_2O_2 (from O_2) and that this effect was enhanced by addition of NAD(P)H to the assay mixture; interestingly, H_2O_2 formation was also stimulated by addition of NAD, malate, Mn^{2+}, and phenolics (such as coniferyl alcohol **4**). In order to explain these observations, it was proposed that the "cell walls" contained a (coupled) wall-bound malate dehydrogenase-peroxidase system which generated superoxide anion and then H_2O_2, with the supply of H_2O_2 being sustained by a malate-oxaloacetate shuttle from the cytoplasm into the cell wall. Importantly, this "cell wall" suspension could also catalyze the formation of "lignin-like" substances when incubated with [^{14}C]-coniferyl alcohol **4**, even when no cofactors were exogenously supplied (GROSS *et al.*, 1977). But, "lignin" yields were approximately doubled by addition of Mn^{2+}, malate and NAD. [In an analogous manner, a wall-bound malate dehydrogenase-peroxidase couple has been proposed for lignifying *Forsythia* xylem cell walls (GROSS and JANSE, 1977).] It must, however, be noted that description of these preparations as "cell walls" is only partly correct, since they undoubtedly contain a myriad of other plant constituents, such as membranaceous materials.

Figure 3. Time course of formation of pinoresinol **1** when [8-^{14}C]coniferyl alcohol **4** was incubated with *F. suspensa* "insoluble" residue. □ = without added cofactors; ■ = with addition of malate (9.5 mM) and NAD (4.5 mM).
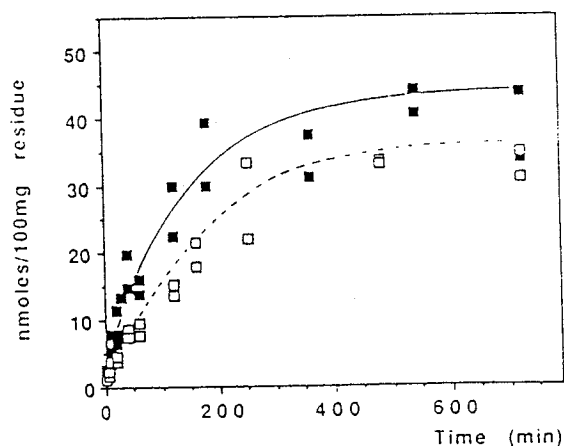
Thus, an insoluble residue was prepared from *Forsythia suspensa* and assayed for its ability to engender (+)-pinoresinol **1a** formation, either without addition of exogenously supplied cofactors or with 9.5 mM malate/4.5 mM NAD supplied (DAVIN *et al.*, 1992). Pinoresinol **1** formation occurred in both instances, although the amounts were significantly increased when malate and NAD were added (Fig. 3). In the absence of exogenously supplied cofactors, it was observed that the ratio of (+)- to (-)-pinoresinol **1a** to **1b** was *ca* 65:35, i.e., that a small but significant stereoselective preference had occurred during coupling. But this stereoselectivity was further enhanced when NAD/malate were added, and the pinoresinol contained > 80% of the (+)-enantiomeric form **1a**. This effect is more clearly understood if we examine the formation of both (+)- and (-)-pinoresinols **1a** and **1b** formed under each assay condition(see Figs. 4a and 4b). As can be seen, the amounts of (-)-pinoresinol **1b** formed is *unaffected* by addition of malate and NAD. [Consequently, it is concluded that the small amounts of (-)-pinoresinol **1b** synthesized are due to non-specific coupling of *E*-coniferyl alcohol **4**; this could be catalyzed by H_2O_2-dependent peroxidase(s), laccase, or polyphenol oxidase(s).

Determining the mechanism of non-specific coupling will be the subject of a future study.] But, when malate and NAD were added, the amounts of (+)-pinoresinol 1a formed were significantly enhanced. This result demonstrated that the stereoselective coupling enzyme required addition of one (or both) cofactors for activity.
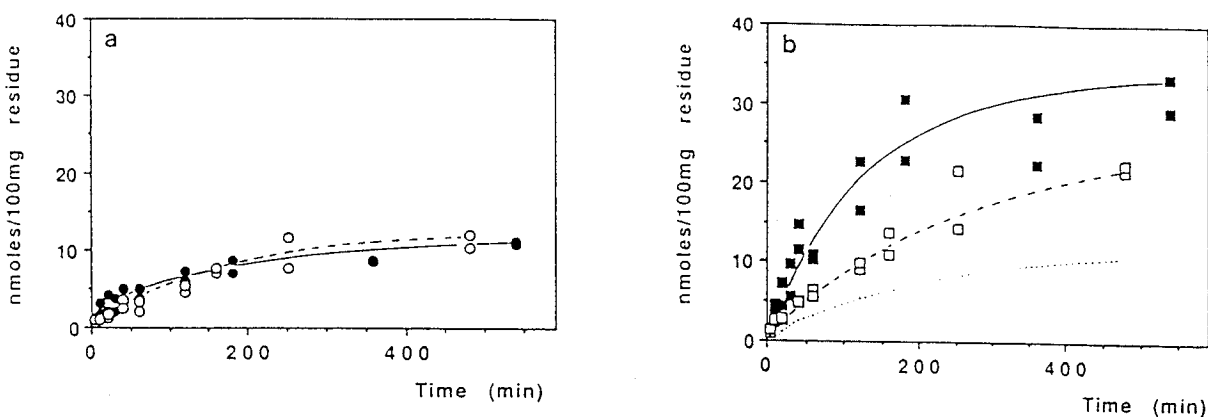

Figure 4. Time course of formation of (a) (-)-pinoresinol 1a and (b) (+)-pinoresinol when [8-14C]coniferyl alcohol 4 was incubated with *F. suspensa* "insoluble" residue. □ = without added cofactors; ■ = with addition of NAD (4.5 mM) and malate (9.5 mM); (- - - -) = contribution to (+)-pinoresinol 1a content due to non-specific coupling.

It was also important to establish whether sinapyl alcohol 8 would undergo a similar stereoselective coupling. But when *E*-sinapyl alcohol 8 was incubated with the *F. suspensa* "insoluble residue", the amounts of syringaresinol 9 formed were unaffected by addition of NAD or malate. Moreover, the syringaresinols 9a/9b were racemic. This suggests that the coupling enzyme does not employ sinapyl alcohol 8 as a substrate, and that racemic syringaresinols 9a/9b are formed via "competing" non-stereoselective coupling.

Lastly, it was of interest to determine whether an insoluble residue from the gymnosperm *Pinus taeda* (loblolly pine) catalyzed the formation of (+)- or (±)-pinoresinols 1a or 1a/1b when incubated with [8-14C]coniferyl alcohol 4; This was of interest because this species does not accumulate significant levels of lignans, and determining stereoselectivity of coupling might shed some light on the lignification process. However, the pinoresinol 1 so obtained was a racemic mixture of pinoresinols 1a/1b. Thus, in an analogous manner to the *Forsythia* investigation, it will be of interest to establish in the future whether this pinoresinol 1a/1b synthesis is catalyzed by laccase, an H2O2-synthesizing system coupled to non-specific peroxidases as proposed by Gross, or polyphenol oxidases. It is anticipated that resolution of this question will have bearing on our understanding of lignification.

Concluding Remarks

Research in this laboratory has established that accumulation of (+)-pinoresinol 1a in *Forsythia* species is due to the action of a highly unusual stereoselective coupling enzyme, catalyzing the coupling of two achiral molecules of *E*-coniferyl alcohol 4. This "insoluble" enzyme preparation requires no exogenously supplied cofactors, but its activity is enhanced when malate and NAD are added; note that low levels of a competing non-specific coupling were also observed, although the precise nature of the enzyme(s) was not investigated. The latter coupling mechanism may be required for the lignification process, but this needs to be resolved. Consequently, future studies will be directed towards solubilization and purification of both stereoselective and non-stereoselective coupling enzymes and their full characterization in order to determine their catalytic mechanisms. Lastly, the (+)-pinoresinol 1a formed by stereoselective coupling then undergoes an enantiospecific enzymatic reduction of the furan ring system to yield (+)-lariciresinol 7a and a second reduction to give (-)-secoisolariciresinol 3b; future work will determine whether more than one enzyme is involved in this highly unusual reduction as well as the mechanism of catalysis.

Acknowledgments

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